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13. Abstract (Maximum 200 Words) <i>(abstract should contain no proprietary or confidential information)</i> Objectives of the project are: (1) to identify the gangliosides of Prostate cancer (CaP) cells that are immunogenic so that they can be used as targets to develop immunotherapy for prostate cancer; (2) to determine the total and specific CaP-gangliosides released into the blood and (3) to assess the nature of immunosuppression induced by CaP gangliosides. The major findings of the first year are as follows: Ganglioside GM1 is the major cell surface ganglioside of normal prostate epithelia. The expression of GM1 is significantly lowered in Prostate cancer cell lines (PC-3, DU145, LNCaP-FGC-10, LNCaP-FGC and HH870). On the other hand, the following gangliosides are more prominent: GM2>GD1b > GT1b (in all cell lines). GD1a is the highly expressed in PC-3 & DU145 cell lines. The immunogenicity of the CaP gangliosides was tested by comparing the serum antibody titers of healthy individuals and CaP-patients against eight different gangliosides. The gangliosides immunogenic in patients are GD1a> GT1b > GM2 > GD1b. The results enable us to identify the pathway of biosynthesis of prostate carcinoma associated gangliosides. The immunogenicity of the gangliosides suggests that they are potential targets for immunotherapy of CaP. The results lead to formulation of allogeneic CaP-vaccine with CaP-gangliosides.			
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Table of Contents

Cover	1
SF 298	2
Table of Contents	3
Introduction	4
Body	5
Key Research Accomplishments	8
Reportable Outcomes	9
Conclusions	9
References	10
Appendices	17

INTRODUCTION

Invasive prostate cancer (CaP) often takes an aggressive course. Prognosis, response to chemo- and hormonal therapy and quality of life are poor for these patients. Therefore, metastatic CaP is considered a candidate for immunotherapy. Developing immunotherapy for CaP requires identifying appropriate target antigens that can be recognized by the immune system. Furthermore understanding the mechanisms by which CaP cells evade the patient's immune mediated attack deserves critical attention. Immunotherapy targeting multiple antigens has not been extended to CaP because of the lack of knowledge about target antigens. Very few correlative studies were made comparing the response to therapy and the presence of other potential target antigens. There is a need to know about the immunogenic targets that can be recognized by cellular or humoral immune system, and that are capable of eliciting immune response. Very few studies have been made on carbohydrate targets of CaP in contrast to the information available on these targets in several solid tumors such as melanoma (1,2), neuroblastoma (3), Sarcoma (4) pancreatic (5) and colorectal carcinomas (6). Gangliosides (Gss) are glycosphingolipids with sialylactosyl ceramide backbone. Although the antigenic domain of the cell surface Gss are smaller than that of proteins, their overexpression and clustered distribution on the tumor cell-surface versus sparse distribution on normal cells makes them potential targets for immune-mediated attack (7).

BODY

This section provides a description of research accomplishments associated with tasks listed in our Statement of Work.

A. Objectives/Hypothesis of this study:

The primary objective of this investigation is to identify the ganglioside-signatures or fingerprints of CaP and establishes the immunogenic gangliosides that can be targeted by immunotherapy. Gangliosides elicit T-cell independent IgM antibody response but not cell mediated immune response. The antibodies thus produced against gangliosides may perform two kinds of functions. One, they may remove the gangliosides released from tumor into circulation. Two, they may bind to tumor cells and mediate complement-dependent cytotoxicity. An important question that should be addressed after identifying the CaP gangliosides is to determine whether they are immunogenic. In simpler terms, we would like to know whether the gangliosides of CaP are recognized by the immune system of patient. Even if they are immunogenic, they may not effectively target the tumor cells, if the gangliosides from tumor cells are released in large quantities. For this purpose, we will be studying whether gangliosides are released from tumor cells into circulation. Total quantity of gangliosides in circulation may vary with tumor burden. If tumor gangliosides change their profile with tumor progression, then the qualitative pattern of gangliosides may vary with stages of disease or tumor progression. Furthermore, gangliosides in circulation are capable of interacting with cytokines to suppress anti-tumor immunosuppression (8). Therefore, the nature of gangliosides in circulation needs to be identified.

B. Task for the first year of the study:

In the first year of the study, our specific aim is to define the ganglioside signatures of human CaP and identify the immunogenic gangliosides that can be used as target antigens for vaccine immunotherapy.

C. Tasks: First year task:

Task # 1 is to determine the nature of gangliosides expressed in human CaP cell lines and biopsied CaP tissues, using established cell lines such as DU145, LNCap-FGC, LNCaP-FGC-10, PC-3 (all ATCC) and HH870 (from Hoag Cancer Center) and six prostate cell lines at JWCI. NCI-H660 was originally included but later deleted because it is not specific for prostate. In addition we proposed to make single cell suspension from 11 cryopreserved CaP biopsy specimens and JWCI cell bank to study the ganglioside profiles.

D. Additional tasks to complete the objectives outlined in the hypothesis:

At the time of submission of the proposal, we have not indicated how we plan to test the immunogenicity of identified CaP-gangliosides.

It has become necessary to identify immunogenicity of the CaP-gangliosides by studying the antiganglioside IgM antibody profiles in cancer patients. This study has become critical, when we realized that the gangliosides released from tumor can augment specific antibodies against CaP-associated gangliosides (8). Indirectly, the anti-

ganglioside IgM titers of different gangliosides provide information on the nature of gangliosides expressed in the tumor of the patients (9). In addition, by comparing the antiganglioside IgM profile of cancer patients at different stages of the disease with that of normal and healthy individuals, we were able to appreciate the unique changes in the profile of the antiganglioside antibodies undergone by the patients during tumor progression.

E. Results: Comparison of Ganglioside signature of normal prostate epithelia or stroma vs cancer cell lines:

It is necessary to compare the ganglioside profile of tumor cells with their respective normal progenitor cells to appreciate the transformation undergone by the cancer cell. Such an approach lead to understanding the unique profile of CaP cells and the synthetic pathway of gangliosides during neoplastic transformation. We have accomplished this investigation using refined monospecific monoclonal antibodies.

F. Results: Identification of CaP-ganglioside signatures or fingerprints:

Figure 1 shows the monospecificity of the monoclonal antibodies used in this investigation to attest the reliability of the results.

Figure 2 shows the ganglioside signatures of selected CaP cell lines and normal progenitor cells.

Figure 3. Biosynthetic pathway of CaP-gangliosides during neoplastic transformation.

G. Results: Evidence of Immunogenicity of CaP-Gangliosides during tumor progression:

Figure 4a & 4B. Profile of antiganglioside IgM antibodies in sera of Stage II (n= 15; A) and Stage IV (n=8) prostate cancer patients.

H. Results: Summary of accomplishments: The salient findings of this study can be enumerated as follows:

1. CaP cell lines express a ganglioside pattern different from that of normal prostate epithelia and stromal cells.
 - The major ganglioside of normal prostate epithelia is the ganglioside GM1 and possibly GM3 (as evidenced by Thin layer chromatographic analyses).
 - All tumor cell lines overexpress GM2, which may arise due to addition of the sugar GalNAc to GM3 by GalNAc transferase activity.
 - Tumor cell lines overexpress GD1a, which may arise due to addition of a sialic acid to terminal galactose of GM1 by 2,3 sialyl transferase.
 - Tumor cell lines also overexpress GD1b, which may arise due to addition of sialic acid to the preexisting sialic acid of GM1 by 2,8 sialyl transferase.
 - GD1a or GD1b may give rise to GT1b.
 - The immunogenic gangliosides in prostate cancer patients are GM2, GD1a, GD1b and GT1.

- Although we observed NeuGc-GM3 in one of the cell lines and no antibodies were detected in the sera of patients, we infer that NeuGc-GM3 might have derived from the fetal calf serum.
- The antibody profiles against these gangliosides differ remarkably between stage II and stage IV patients as indicated in Figure 4. While GD1a and GT1b are more immunogenic in the early stage of the cancer, GM2 and GD1b are found to be immunogenic in stage IV patients. This difference signifies the change in the profile of gangliosides released during tumor progression. In Task 2 this aspect will be focussed.
- These four gangliosides should serve as ideal targets for immunotherapy of CaP.
- The augmentation of immune response in Prostate cancer patients can be achieved by immunization of allogeneic prostate cancer cell lines rich in these gangliosides together with an immunostimulant.

I. **Results: Problems in accomplishing the tasks:** We have encountered the following problems while accomplishing the task.

1. Availability of Biopsies is much restricted. Even if available the volume of tumor tissue is too small to permit a detailed biochemical analyses.

Solution: We plan to pool tumor biopsies to characterize the ganglioside profile of CaP.

2. Paraffin sections of CaP biopsies are not useful for immunostaining of gangliosides, because gangliosides are lost due to phase-transition or membrane solubility at temperature above 42°C.

Solution: We plan to obtain frozen or cryocut sections of tumor biopsies from our collaborators.

3. Although the ganglioside is identified to be GM1 in normal prostate epithelia, we need to distinguish whether it is GM1a or GM1b. For this purpose, we need standard GM1b and monoclonal antibody to GM1b.

Solution: We have approached Dr. Jacques Portoukalian, at University of Lyons, France to solve this problem. He has kind enough to give us monoclonal antibodies for GM1b. In addition, he has suggested that a murine cell line called YAC-1 has very high level of GM1b for extraction. We plan to extend the task to complete these studies.

J. **Results: Recommended Changes based on the accomplished data:** In order to complete the task defined for first year, we intend to do the following:

1. To establish the changing profile of CaP-gangliosides during tumor progression, we consider it imminent to analyze sera of different stages of CaP patients for fingerprint of antiganglioside antibodies. We plan to increase the sample size to obtain statistically validated conclusions. This study may overlap task 2 to be undertaken in year # 2.

2. Grow YAC-1 cells for obtaining GM1b and test whether the CaP cells express GM1b. GM1b is identified as the potent

progenitor of GD1a and GT1b. This finding remains to be confirmed because normal extraneural human tissues do not express GM1b and possible it is an important progenitor ganglioside of CaP gangliosides.

3. One essential requirement of biochemical analyses of gangliosides Using TLC is uniform spotting of gangliosides on TLC. On recommendation of Dr. Jacques Portoukalian our foreign collaborator, our institute has purchased a LINOMAT TLC spotting equipment with N2 cylinders, in the month of July. We have started our biochemical analysis of the cell lines and tumor biopsies.
4. In order to obtain tumor biopsies and sera for this study, we have approached to Surgeons who operate on prostate cancer patients.
 - Dr. Stanley Grossman is at Saint John Hospital and UCLA Santa Monica Hospital. He has agreed to provide tumor tissues and sera or plasma samples from different stages of the patients to complete our tasks. He has been included in our protocol submitted to Institutional Review Board. I have been included in Grossman's study submitted to IRB.
 - Dr. Rajvir Dahiya, Department of Urology, University of California, San Francisco V.A. Medical Center has kindly consented to provide serum samples from different stages of the patients to complete our tasks.
5. In addition, I have requested Dr. Jacques Portoukalian, Director of Research, INSERM U. 346, Hospital Edouard Herriot, 69437 Lyon, Cx 03 France, to be a consultant in this project to advise on characterizing the ganglioside fingerprints of prostate cancer. His role will be restricted to advise in methodologies and analyses of the results. He will not provide tissue or serum samples.
6. Dr. R.S. Selvan, Senior Scientists from Vaccine Research Laboratory at Hoag Cancer Center, New Port Beach, California and Dr. Ralph Jones at Uniformed Services Maryland, Bethesda will remain as active collaborators in this project.
7. We plan to complete the confirmatory experiments before submission of the manuscript by the end of this year.
8. The manuscript will be entitled tentatively as "Signature of Immunogenic Gangliosides of Prostate Carcinoma."

KEY RESEARCH ACCOMPLISHMENTS

Key research findings emanating from this investigation can be listed as follows:

- Normal Prostate Epithelia contains GM1 and GM3, whereas neoplastic transformation results in conversion of these gangliosides into GM2, GD1a, GD1b and GT1b in CaP cell lines.
- The Signature or fingerprint of gangliosides of CaP can be defined as GM2>GD1a>GD1b>GT1b.
- All these gangliosides are immunogenic in prostate cancer patients.
- Antibodies to GD1a and GT1b are most prevalent in the sera of stage II prostate cancer patients, whereas antibodies to GM2 and GD1b, in addition to that of GD1a and GT1b, are prevalent in stage IV

prostate cancer patients, suggesting either change in profile of CaP-associated gangliosides or their release into circulation during tumor progression.

REPORTABLE OUTCOMES

1. A part of the results of this investigation is submitted as an abstract for the Immunology & Immunotherapy Workshop 2002, to be held in Havana, Cuba. The abstract is enclosed (Appendix # 1).
2. A Manuscript entitled "Signature of Immunogenic Gangliosides of Prostate Cancer" is under preparation.
3. Based on the results of this investigation, we have obtained funds from the Board of Directors of John Wayne Cancer Institute to purchase a Linomat TLC spotter (\$ 6,500) and a Shimadzu HPLC (\$ 35,000) to purify the minute quantities of gangliosides from prostate cancer. The instruments were installed in our laboratory in July.
4. The Board of Directors of John Wayne Cancer Institute has also provided funds for the visit of Dr. Jacques Portoukalian for 2 months to provide necessary advice and consultation. Jacques is well versed in Shimadzu HPLC of gangliosides. He has gone through the results and agreed to be a co-author in the abstract and the manuscript.

CONCLUSIONS

In this project we have identified gangliosides as Prostate carcinoma associated antigens. Based on our experience with gangliosides of several kinds of solid tumors, we find the gangliosides of CaP are unique. The ganglioside profile of CaP differs from that of normal prostate epithelia and stromal cells. The major ganglioside of normal prostate epithelia is the ganglioside GM1 and possibly GM3 (as evidenced by thin layer chromatographic analyses). All tumor cell lines overexpress GM2, which may arise due to addition of the sugar GalNAc to GM3 by GalNAc transferase activity. Tumor cell lines overexpress GD1a or GD1b, which may arise due to addition of a sialic acid to terminal galactose of GM1 by 2,3 sialyl transferase or a sialic acid to the preexisting sialic acid of GM1 by 2,8 sialyl transferase, respectively. All these gangliosides are immunogenic in CaP patients as evidenced by antibody response. The antibody profiles against these gangliosides differ remarkably between stage II and stage IV patients. While GD1a and GT1b are more immunogenic in the early stage of the cancer, GM2 and GD1b are found to be immunogenic in stage IV patients. There is a need to confirm the findings on CaP-associated gangliosides with additional experiments and data involving tumor biopsies and cell lines. Furthermore, the study of serum gangliosides in second and subsequent tasks to be carried out in the second year will shed light on the nature of gangliosides released into tumor microenvironment that promote immunosuppression.

"So What Section"

This study establishes that gangliosides, the sialic acid containing glycolipids are important prostate cancer-associated-antigens and can serve as targets antigens for immunotherapy. Since gangliosides are known to cause immunosuppression in patients, the release of gangliosides from tumors into tumor microenvironment or blood during tumor progression may promote immunosuppression. This difference observed

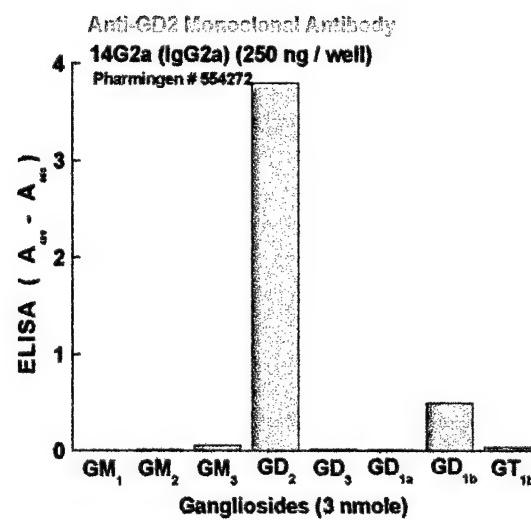
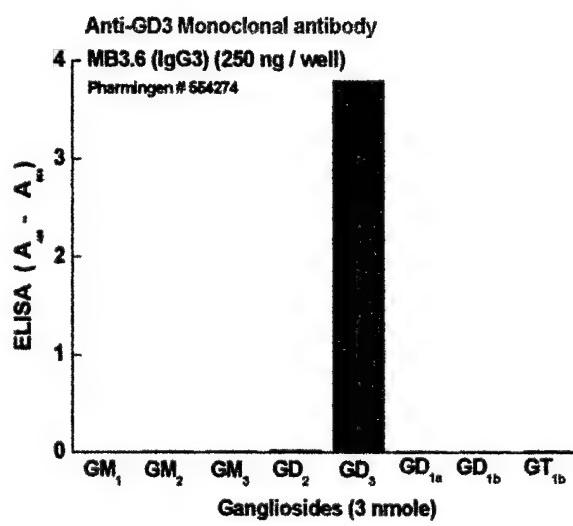
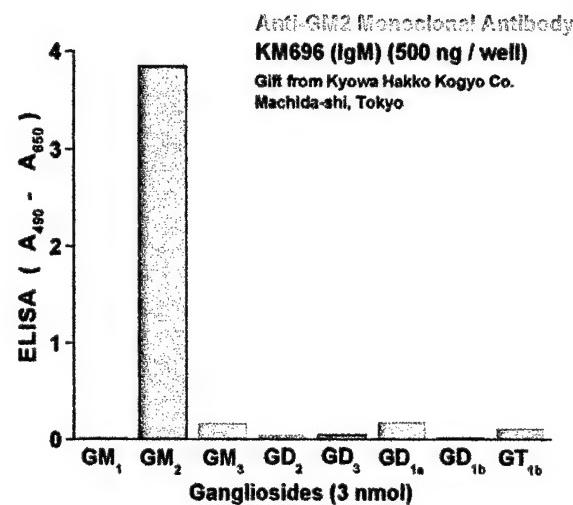
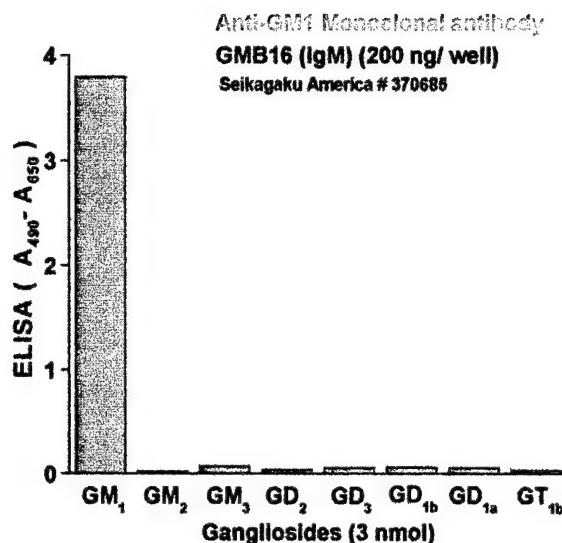
in the antiganglioside antibody profiles in sera of stage II and IV patients signifies the change in the profile of gangliosides released during tumor progression. Based on the pattern of gangliosides, we can trace the sequence of immunosuppression in these patients. This will be the task for third year. However, in the second year, we will establish the nature of gangliosides released into circulation. Once we know their nature, then we can design experiments to elucidate the nature of immunosuppression.

In addition, our results suggest that these four gangliosides should serve as ideal targets for immunotherapy of CaP. The augmentation of immune response in Prostate cancer patients can be achieved by immunization of allogeneic prostate cancer cell lines rich in these gangliosides together with an immunostimulant. The finding supports our main objective of developing an active specific immunotherapy for prostate cancer. The active specific therapy against cancer involves removal of immunosuppressive gangliosides from patients' tumor microenvironment and circulation using antiganglioside antibodies. Elimination of immunosuppression and restoration of immune competence is the primary role of active specific immunotherapy. After restoration of immunocompetence, the patients' residual tumor can be targeted by humoral and cell mediated immunity. Gangliosides play a role in both these functions of active specific immunotherapy.

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Figure 1. The monospecificity of the monoclonal antibodies against gangliosides used in this study.



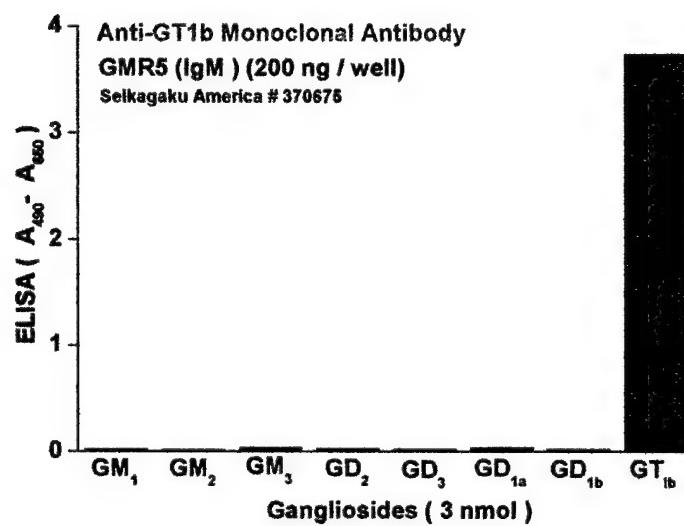
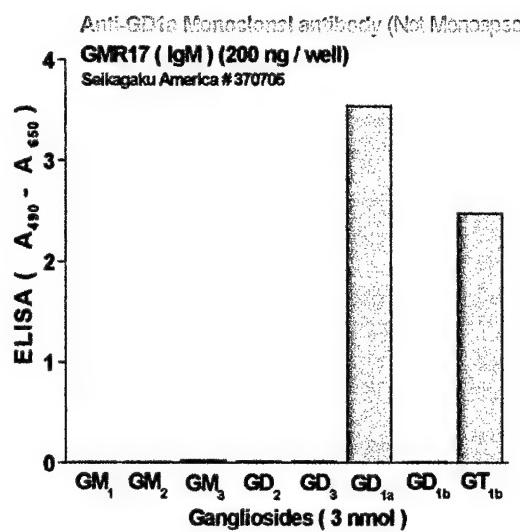
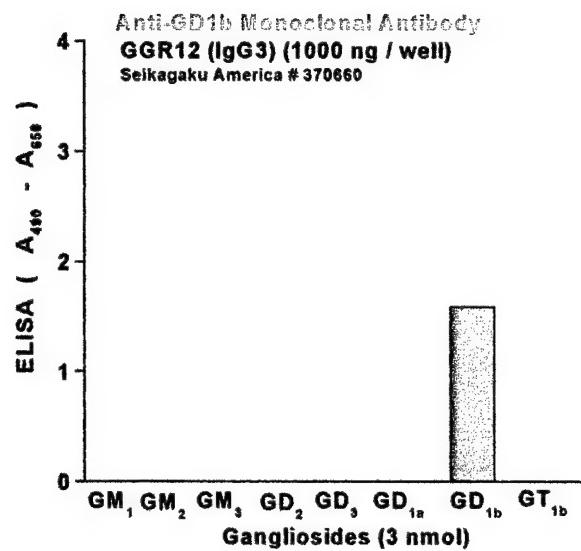
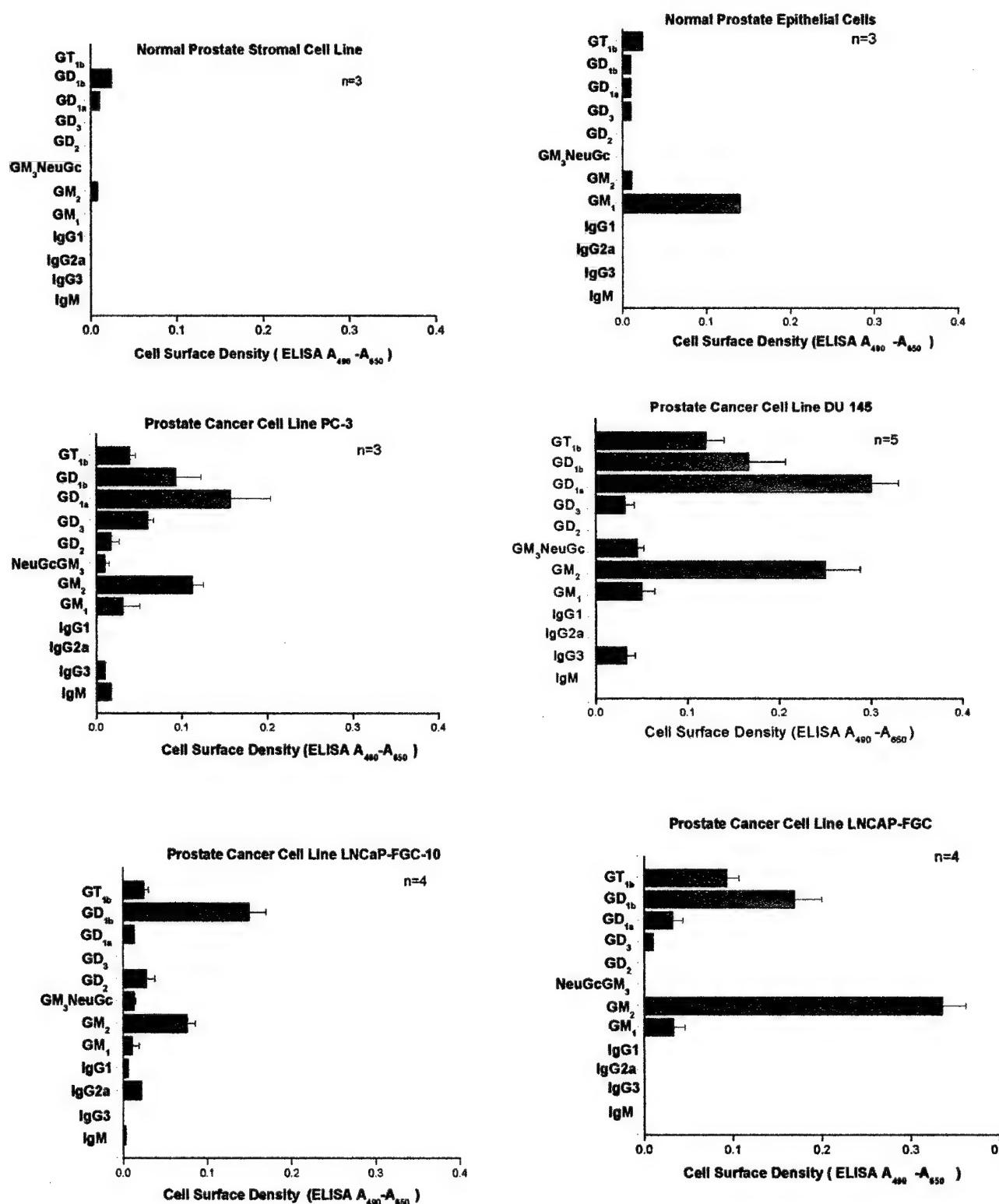


Figure 2. The ganglioside signatures of normal progenitor cells (2 cell lines) selected CaP cell lines (5 Cell lines). Isotype controls of monoclonal antibodies are shown as negative control.



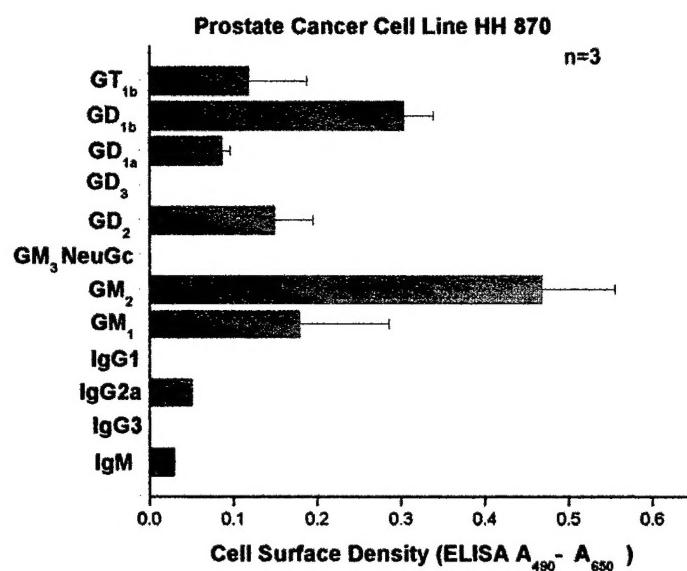


Figure 3. Signature and Biosynthetic Pathway of Prostate Carcinoma Gangliosides during neoplastic transformation.

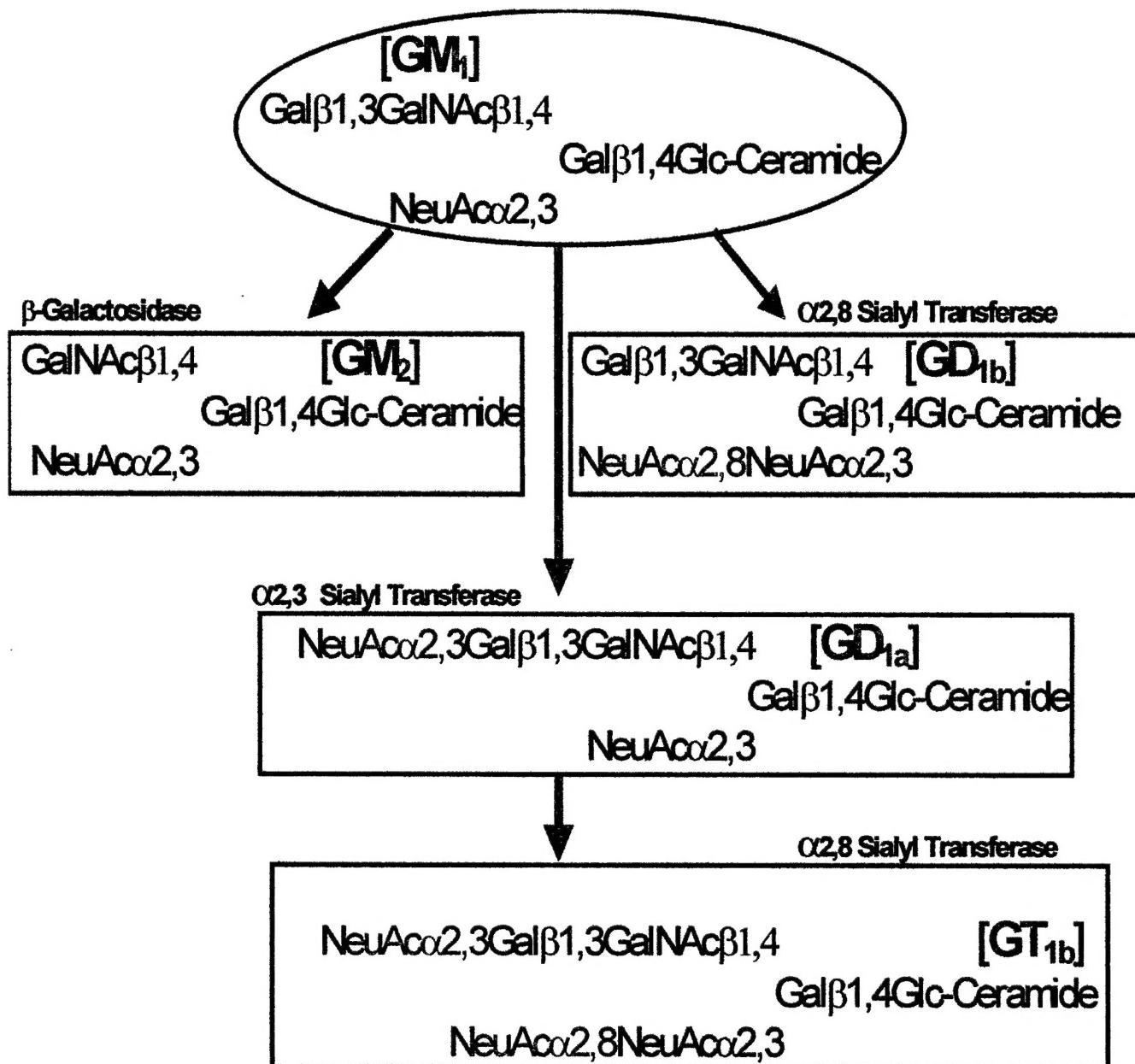
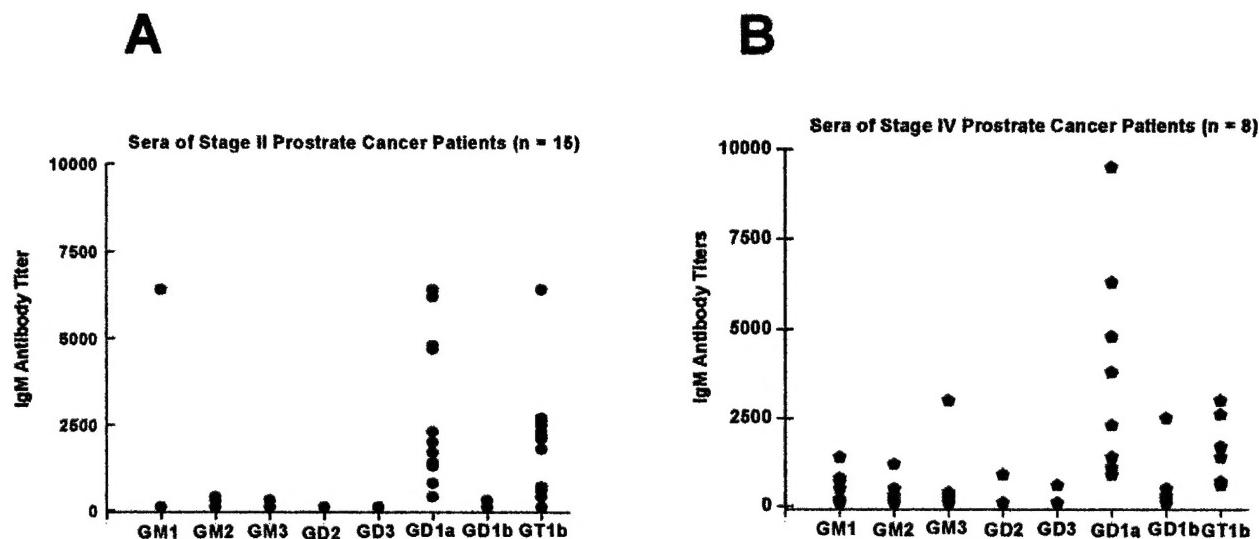


Figure 4. Profile of antiganglioside IgM antibodies in sera of Stage II (n= 15; A) and Stage IV (n=8) prostate cancer patients. Note prevalence of anti-GD1a and anti-GT1b IgM antibodies in these patients.



APPENDICES

Evidence for IgM response to GD1a and GT1b in patients with early stage Prostate carcinoma and Melanoma

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Using specific monoclonal antibodies to measure cell surface density of different gangliosides in human prostate carcinoma cell lines with ELISA system, we have examined the expression of gangliosides in five prostate cancer cell lines. Most of the cell lines expressed high density of GM2, GD1a, GD1b and GT1b on the tumor cell surface. We have screened the sera of 14 prostate cancer patients (TNM stage T1c) for antibodies against GM3, GM2, GM1, GD3, GD2, GD1b, GD1a and GT1b using a sensitive ELISA. None of the patients showed IgG antibodies to any of the gangliosides. While the IgM titers of GD1a and GT1b were very high and ranged between 400 and 6400, the IgM titers against other gangliosides remained low, suggesting that the major prostate carcinoma-associated gangliosides GD1a and GT1b signaled antibody production in these patients. Although GM2 and GD1b are found on tumor cell surface, the serum titers against these gangliosides were low in most of the patients. Sera of Stage III melanoma patients were used as positive controls, which showed high titers of IgM to GD3, GD2, GM2 and GD1b.

While IgG antibodies to gangliosides are low or negligible, the profiles of IgM antibodies are most prevalent in the sera of melanoma patients (TNM stages T1a/b & T2a/b). The titers were high only for anti-GD1a and anti-GT1b IgM antibodies. Although GD1a and GT1b were reported in melanoma tumor biopsies and cell lines, the antibody response in early stage of the disease was intriguing. We have compared the serum anti-GD1a and GT1b IgM titers of the patients who had recurrent disease and expired (EXP) after surgical resection of the primary (median survival time 23.9 months) with those who are alive and have no evidence of disease (NED) (median follow up time 203.4 months) within 6 months after surgery. We found that the titers of anti-GD1a IgM ($p < 0.01$) and anti-GT1b IgM ($p < 0.01$) were significantly higher in patients who expired due to recurrent disease as compared to those with NED, suggesting that anti-GD1a and anti-GT1b IgM antibodies are poor prognosticators of stage T1 & T2 melanoma with or without ulceration.

In this study, we have identified, for the first time, the gangliosides GD1a and GT1b as important immunogenic gangliosides of early stages of prostate cancer and melanoma. GD1a and GT1b are known as potent immunosuppressive gangliosides, the former induces production of IL-10. Probably, IgM antibodies are produced during early stages of the disease to clear them from circulation to prevent immunosuppression.

Supported by Defense Prostate Cancer Research Program (Grant No: DAMD17-01-1-0062) of the U.S. Army Medical Research and Materiel Command of CDMRP and State of California, Department of Health Services (TTP1020) for Melanoma Research.